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Attorney Docket P1363R1
PATENT

Jc525 U.S. PTO
09/320100
05/26/99

CERTIFICATION UNDER 37 CFR 1.10	
EM 168 883 029, US Express Mail Number	May 26, 1999, Date of Deposit
I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.	
 Pamela Gavette	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION
Assistant Commissioner of Patents
Washington, D.C. 20231

NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Deborah Ann Ansaldi
Philip Lester

Title: **SEPARATION OF POLYPEPTIDE MONOMERS**

1. Type of Application

This application is for an original, non-provisional application.
 This is a non-provisional application claiming priority to provisional application no. 60/087,602, filed June 1, 1998, the entire disclosure of which is hereby incorporated by reference.
 This is a [] continuation-in-part [] continuation [] divisional application claiming priority to application Serial Number_____, filed ____, the entire disclosure of which is hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b)
(Non-provisional)**

14 pages of specification
2 pages of claims
1 page(s) of abstract
6 sheet(s) of drawings
[x] formal [] informal

3. Declaration or Oath

(for new and CIP applications; also for Cont./Div. where inventor(s) are being added)

An executed declaration of the inventor(s) [] is enclosed [x] will follow.

(for Cont./Div. where inventorship is the same or inventor(s) being deleted)

A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

(for Cont./Div. where inventor(s) being deleted)

A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

4. Assignment

(for new and CIP applications)

An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [x] will follow.

(for cont./div.)

The prior application is assigned of record to Genentech, Inc.

5. Amendments (for continuation and divisional applications)

Cancel in this application original claims ___ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119

Amend the specification by inserting before the first line the sentence:

--This is a

non-provisional application
 continuation
 divisional
 continuation-in-part

of co-pending application(s)

Serial No. __ filed on __, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120. --

International Application __ filed on __ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC

§120.--

_____ provisional application No. _____ filed ___, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--.

6. Fee Calculation (37 CFR 1.16)

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$760.00
Total Claims	13	- 20 =	0	X \$18.00	\$0.00
Independent Claims	1	- 3 =	0	X \$78.00	\$0.00
Multiple dependent claim(s), if any				+ \$260.00	\$0.00
Filing Fee Calculation					\$760.00

7. Method of Payment of Fees

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$760.00. A duplicate copy of this transmittal is enclosed.

8. Authorization to Charge Additional Fees

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. A duplicate copy of this sheet is enclosed.

9. Additional Papers Enclosed

- Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- A new Power of Attorney or authorization of agent.
- Other:

10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)

[This item must be completed and the necessary papers filed in the prior application if the period set in the prior application has run]

- _____ A petition, fee and/or response has been filed to extend the term in the pending prior application until
- _____ A copy of the petition for extension of time in the **prior** application is attached.

11. Correspondence Address:

X Address all future communications to:

GENENTECH, INC.
Attn: Janet E. Hasak
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Respectfully submitted,
GENENTECH, INC.

Date: May 26, 1999

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SEPARATION OF POLYPEPTIDE MONOMERS

10

Background of the Invention

Related Applications

This application is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional application number 60/087,602 filed June 1, 1998, the contents of which are incorporated herein by reference.

15 Field of the Invention

This invention relates to a process for separating polypeptide monomers from dimers and/or other multimers using ion-exchange chromatography.

Description of Background and Related Art

Attempts to purify authentic, properly folded protein from recombinant hosts have been frustrated due to the tertiary structure of the molecule. In this regard, purification of the recombinantly produced molecule often yields a heterogeneous mixture that consists largely of inactive, misfolded, insoluble, and/or soluble dimers, multimers, and disulfide-linked aggregates. Other aberrant molecules, such as fragments, nicked, oxidized, and glycosylated forms, may also be present. Thus, purification is difficult and yields of the authentic monomer are often low. See, e.g., Elliott *et al.*, *J. Protein Chem.*, 9: 95-104 (1990).

Different techniques have been used to correct these problems. For example, Chang and Swartz, *Protein Folding: in vivo and in vitro* (American Chemical Society, 1993), pp. 178-188 describe a method for solubilizing aggregated IGF-I produced in *E. coli*, using low concentrations of urea and dithiothreitol (DTT) in an alkaline buffer. U.S. Pat. No. 5,231,178 describes a method for the purification of correctly folded, monomeric IGF-I from *P. pastoris* using a combination of cation exchange, hydrophobic interaction, and gel filtration chromatography. WO 96/40776 describes a method for producing authentic properly folded IGF from yeast using a first cation exchange chromatography with the yeast cell medium, denaturing and chromatography, and performing reverse phase high performance liquid chromatography.

Separation of protein and peptide monomers from their dimers, tetramers, and multimers presents a serious challenge to the separations scientist. Size-exclusion chromatography (SEC) and Tangential-Flow Filtration (TFF) (U.S. Pat. Nos. 5,256,294 and 5,490,937) have been used for separating monomers from aggregates but have limitations. SEC can separate monomers from multimers, and in 5 some cases monomers from dimers. The main limitations of SEC are 1) limited load volumes (typically 5% of the bed volume) requiring large columns or multiple cycles, 2) and load protein concentration (low concentration feed stocks require pre-concentration or multiple cycles on the column. Higher protein concentrations can be more viscous, thereby reducing the efficiency of the separation). Historically TFF can separate protein multimers that are ten-fold larger than the monomer. U.S. Pat. No. 10 5,256,294.

U.S. Pat. Nos. 4,228,154 and 5,250,663 disclose separations of albumin from mixtures. U.S. Pat. No. 4,228,154 describes use of both cation-exchange and anion-exchange chromatography steps for the purification, without separation of monomer from multimers.

There is a need for separating monomers from dimers and multimers that is satisfactory, requires the use of only one ion-exchange step, and does not have the limitations of SEC or TFF.

Summary of the Invention

Accordingly, this invention provides a method for separating a polypeptide monomer from a mixture comprising dimers and/or multimers, wherein the method comprises applying the mixture to either a cation-exchange or an anion-exchange chromatography resin in a buffer, wherein if the resin is cation-exchange, the pH of the buffer is about 4-7, and wherein if the resin is anion-exchange, the pH of the buffer is about 6-9, and eluting the mixture at a gradient of about 0-1 M of an elution salt, wherein the monomer is separated from the dimers and/or multimers present in the mixture.

In this study it is demonstrated that ion-exchange chromatography—either anion or cation—is an effective means to separate protein or polypeptide monomers from their dimers and/or multimers. Separations are performed using either step or linear gradient elution. Ion exchange has several advantages over the SEC and TFF methods described above. First, separation is independent of polypeptide concentration in the load and therefore no pre-concentration is required. Second, resins can be loaded to greater than 30 mg polypeptide/mL resin and still achieve excellent separations. Third, ion-

exchange resins are inexpensive and easy to use. Typical separations achieve enrichment of monomer to greater than 99.5% purity and yields in excess of 90%.

Brief Description of the Drawings

5 Figures 1A and 1B show separation of U266 IgE monomer from dimers and multimers on a RESOURCE QTM anion-exchange column. The column was equilibrated in 25 mM Tris/pH 8, and eluted with a gradient from 0 to 0.5 M sodium chloride over 10 column volumes. Fig. 1A is full-scale; Fig. 1B is a close-up view to show the dimers and multimers.

10 Figures 2A1, 2A2, 2B, and 2C show separation of anti-IgE monoclonal antibody monomer from dimers and multimers. Figs 2A1 and 2A2 were run on a RESOURCE QTM anion-exchange column. Fig. 2A1 is full-scale; Fig. 2A2 is a close-up view to show the dimers and multimers. Fig. 2B is a run on Q-SEPHAROSE FAST-FLOWTM resin. Fig. 2C is a plot of monomer and dimer/multimer observed in fractions, where the open dots are monomer and the solid dots are dimer. The monomer and dimer/multimer were determined using a SUPERDEX 200 HRTM 10/30 analytical size-exclusion column (Pharmacia Biotech). In all cases the columns were equilibrated in 25 mM Tris/pH 8. The gradient used in the Fig. 2A panels was 0 to 0.5 M sodium chloride over 40 column volumes. The gradient used for Fig. 2B (Q-SEPHAROSE FAST-FLOWTM) was 0.05 to 0.2 M NaCl over 10 column volumes.

15 Figures 3A-C show separation of BSA monomer and dimer on a RESOURCE QTM anion-exchange column at pH 8. The column was equilibrated in 25 mM Tris/pH 8, and eluted with a gradient from 0.125 to 0.275 M sodium chloride over 40 column volumes. Fig. 3A is purified monomer, Fig. 3B is purified dimer, and Fig. 3C is a commercial preparation of BSA (Bayer) that contains both monomer and dimer.

20 Figures 4A-C show separation of BSA monomer and dimer on a RESOURCE QTM anion-exchange column at pH 6. The column was equilibrated in 20 mM sodium phosphate/pH 6, and eluted with a linear gradient from 0 to 0.5 M sodium chloride over 10 column volumes. Fig. 4A is purified monomer, Fig. 4B is purified dimer, and Fig. 4C is a commercial preparation of BSA (Bayer) that contains both monomer and dimer.

25 Figures 5A and 5B show separation of anti-IgE monoclonal antibody monomer from dimers and multimers on a RESOURCE STM cation-exchange column at pH 6. The column was equilibrated in 20

mM sodium phosphate/pH 6, and eluted with a linear gradient from 0 to 0.05 M sodium chloride over 30 column volumes. Fig. 5A is the chromatogram from the separation, and Fig. 5B is a plot of monomer and dimer/multimer observed in fractions using the same method described in Figure 2, where the open dots are monomer and the solid dots are dimer.

5 Figures 6A and 6B show separation of BSA monomer and dimer on a RESOURCE S™ cation-exchange column at pH 4.3. The column was equilibrated in 20 mM sodium acetate/pH 4.3, then eluted with a gradient from 0 to 1 M sodium chloride over 20 column volumes. Fig. 6A is purified monomer, and Fig. 6B is purified dimer.

10

Detailed Description of the Preferred Embodiments

Definitions

As used herein, "polypeptide" refers generally to peptides and proteins having more than about ten amino acids. Preferably, the polypeptides are "exogenous," meaning that they are "heterologous," *i.e.*, foreign to the host cell being utilized, such as a human protein produced by *E. coli*. However, they may also be derived from a native source in which they are present naturally.

Examples of mammalian polypeptides include molecules such as, *e.g.*, renin, a growth hormone, including human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; 1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; thrombopoietin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; a serum albumin such as human serum albumin; mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as brain-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF; cardiotrophins (cardiac hypertrophy factor) such as cardiotrophin-1 (CT-1); platelet-derived

growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF- 1, TGF- 2, TGF- 3, TGF- 4, or TGF- 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD-3, CD-4, CD-8, and CD-19;

5 erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; serum albumin, such as human serum albumin (HSA) or bovine serum albumin (BSA); colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; anti-HER-2 antibody; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a

10 portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; and fragments of any of the above-listed polypeptides.

The preferred polypeptides of interest are mammalian polypeptides. Examples of such mammalian polypeptides include enzymes, hormones, cytokines, albumins, chemokines, immunotoxins, viral components, antibodies, neurotrophins, and antigens. Suitable such polypeptides encompass polypeptides such as HSA, BSA, anti-IgE, anti-CD20, anti-IgG, t-PA, gp120, anti-CD11a, anti-CD18, anti-VEGF, VEGF, TGF-beta, activin, inhibin, anti-HER-2, DNase, IGF-I, IGF-II, brain IGF-I, growth hormone, relaxin chains, growth hormone releasing factor, insulin chains or pro-insulin, NGF, NT-3, BDNF, and urokinase. Particularly preferred mammalian polypeptides include, *e.g.*, t-PA, gp120 (IIIb), anti-HER-2, anti-CD11a, anti-CD18, anti-VEGF, VEGF, BSA, HSA, anti-CD20, anti-IgE, anti-IgG, DNase, IGF-I, IGF-II, TGF-beta, IGFBP-3, IGFBP-2, IGFBP-1, growth hormone, NGF, NT-3, NT-4, NT-5, and NT-6. The polypeptide is more preferably an antibody or a serum albumin, more preferably, anti-IgE, anti-IgG, anti-Her-2, anti-CD11a, anti-CD18, anti-CD20, anti-VEGF, BSA, or HSA.

For purposes herein, the “mixture” contains monomers and either dimers or multimers or both dimers and multimers. Typically, the mixture is a biological fluid, which denotes any fluid derived from or containing cells, cell components, or cell products. Biological fluids include, but are not limited to, fermentation broth, cell culture supernatants, cell lysates, cleared cell lysates, cell extracts, tissue extracts, blood, plasma, serum, sputum, semen, mucus, milk, and fractions thereof. This definition includes cell-conditioned culture medium, which denotes a nutrient medium in which cells have been cultured and which contains cell products.

For purposes herein, "ion-exchange chromatography resin" refers to chromatography medium for anion- or cation-exchange separation.

As used herein, "elution salt" refers to an alkaline earth, alkali metal, or ammonium salt, i.e., a salt having a cation from the alkaline earth or alkali metal elements or an ammonium cation and having
5 an inorganic or organic (hydrocarbon-based) anion. Examples of such salts include sodium chloride, ammonium chloride, sodium citrate, potassium citrate, potassium chloride, magnesium chloride, calcium chloride, sodium phosphate, calcium phosphate, ammonium phosphate, magnesium phosphate, potassium phosphate, sodium sulfate, ammonium sulfate, potassium sulfate, magnesium sulfate, calcium sulfate, etc. Preferred salts herein are chlorides or sulfates. The most preferred salt herein is sodium
10 chloride.

As used herein, "multimers" refer to n-mers where n is 3-10, i.e., polymers that are not dimers but exclude aggregates. In contrast to multimers, aggregates have a value for n of greater than 10, and/or a molecular weight of greater than 2 million daltons, and/or are species contained in the excluded volume of analytical size-exclusion chromatography columns such as SUPEROSE 6TM (Pharmacia).

Modes for Carrying out the Invention

This invention relates to a method of separating monomers of polypeptides from their dimers or multimers or both. The method involves placing the mixture of monomers and dimers and/or multimers, from whatever source, in an equilibration buffer at a pH in the range of about 4 and 9 depending on whether the resin used for chromatographic separation is a cation- or anion-exchange resin. The resulting mixture is loaded onto either a cation-exchange or anion-exchange chromatography resin to capture all the n-mers (monomers, dimers, trimers, tetramers, etc.) present in the mixture. For ion-exchange column chromatography, ligands of general affinity can be used to achieve the desired selectivities and binding properties. The loading takes place in a buffer at a pH of about 6-9 if the resin is anion-exchange and about 4-7 if the resin is cation-exchange. The exact pH will depend, for example,
25 on the isoelectric point of the polypeptide.

If the resin is a cation-exchange resin, prior to loading the mixture, the matrix can be equilibrated using several column volumes of a dilute, weak acid (e.g., four column volumes of 20 mM acetic acid, pH 3, or of 20 mM phosphoric acid, pH about 2.8). Following equilibration, the mixture is added and the column can be washed one to several times, prior to elution of the mixture, also using a weak acid

solution such as a weak acetic acid or phosphoric acid solution. The buffer used for this purpose depends on, e.g., the polypeptide and the anionic or cationic nature of the resin. For anion-exchange, preferably the buffer is TRIS or phosphate buffer; for cation-exchange, the buffer is preferably acetate or phosphate buffer.

5 Ion-exchange chromatography is typically carried out at a temperature of about 18-25°C, preferably about 20°C (room temperature). The preferred column loading is about 1 ml resin per 20-30 mg total polypeptide.

10 Following adsorption of the n-mer molecules to the ion exchanger, the mixture is eluted by contacting the resin with an elution salt having an appropriate ionic strength to displace the monomer from the matrix. An elution salt gradient is used of about 0 to 1 M. The gradient may be linear or stepwise. Preferably the gradient is from about 0 to 500 mM elution salt, more preferably 50 to 200 mM elution salt, and most preferably, 0 to 50 mM elution salt. Preferably the elution salt is a sodium salt, such as sodium chloride, although other elution salts and concentration gradients, known to those of skill in the art, also find use herein. The quantity of elution buffer can vary widely and will generally be in the range of about 2 to 40 column volumes, preferably 10 to 40 column volumes. Following elution, the eluate can be assayed for total monomeric concentration.

20 Suitable cation-exchange resins herein include a wide variety of materials known in the art, including those capable of binding polypeptides over a wide pH range. For example, carboxymethylated, sulfonated, agarose-based, or polymeric polystyrene/divinyl benzene cation-exchange matrices are particularly preferred. Other useful matrix materials include, but are not limited to, cellulose matrices, such as fibrous, microgranular, and beaded matrices; dextran, polyacrylate, polyvinyl, polystyrene, silica, and polyether matrices; and composites. These matrices include, for example, CM52 CELLULOSE™ (Whatman, Inc.); S-HYPERD™ and CM SPHERODEX™ (Secpracor); SP SEPHAROSE FF™, DEAE SEPHAROSE FF™, CM-SEPHAROSE™, and RESOURCE S™ (Amersham Pharmacia Biotech AB);
25 and JT BAKER CSx™ (J.T. Baker, Inc.), as well as those containing the functional ligand R-SO₃⁻, preferably sulfopropyl resins, such as TOYOPEARL SP550C™ (Tosohas) and FRACTOGEL EMD™ SO₃⁻ -650 (m) (Merck). Other suitable materials for use in cation-exchange chromatography are within the knowledge of those skilled in the art.

Anion-exchange chromatography is carried out using media appropriate therefor, as are known in the art. Suitable media include, e.g., polymeric polystyrene/divinyl benzene resins and agarose-based resins, as well as agarose beads, dextran beads, polystyrene beads, media that comprise an insoluble, particulate support derivatized with tertiary or quaternary amino groups., and supports derivatized with 5 trimethylaminoethyl groups. Examples of suitable such media include DE92TM (diethylaminoethyl cellulose, Whatman); DEAE-CELLULOSETM (Sigma), BAKERBOND ABX 40 muTM (J.T. Baker, Inc.); DEAE resins such as FRACTOGEL EMD DEAE-650TM (EM Separations), FRACTOGEL EMD TMAE-650 (S)TM (EM Science, Gibbstown, NJ), TSK gel DEAE-SPWTM (Tosohaa), DEAE-SEPHAROSE CL-6BTM and chelating SEPHAROSETM (Amersham Pharmacia Biotech AB), DEAE MERE SEP. 1000TM 10 (Millipore), and DEAE SPHERODEXTM (Sepracor); RESOURCE QTM and Q SEPHAROSETM (QSFF) (Amersham Pharmacia Biotech AB); MACRO-PEP QTM (Bio-Rad Laboratories, Hercules, CA); Q-HYPERDTM (BioSeptra, Inc., Marlborough, Mass); and the like. Other suitable anion-exchange chromatography materials, as well as the selection and use of these materials for the present application, are conventional in the art.

Purified fractions of monomer obtained from the ion-exchange chromatography may be further processed by subjecting them to any appropriate technique designed for downstream processing and purification. This will depend largely on the type of polypeptide and its intended use. Only one ion-exchange step is necessary to effect the desired separation of monomer from dimers and/or multimers in a mixture, although the invention does not exclude using more such steps if desired in the upstream or downstream processing of the polypeptide.

The invention will be more fully understood by reference to the following examples. They should not, however, be construed as limiting the scope of the invention. All literature and patent citations herein are incorporated by reference.

25

EXAMPLE I

This example shows the separation of anti-IgE monomers and bovine serum albumin monomers from dimers and multimers.

MATERIALS AND METHODS:

Resins:

Pharmacia Q-SEPHAROSE FAST FLOW™. 4mL to 235L bed volumes evaluated

Pharmacia RESOURCE S and RESOURCE Q™: 1mL prepacked columns

5 JT Baker CSx™, 0.46 X 5cm, 5 μ particles

Proteins:

A. Humanized anti-IgE monoclonal antibodies (IgG₁) available from Genentech, Inc.: pI ~ 7.5, designated as E25 and E26. WO 93/04173 published March 4, 1993 describes humanized anti-IgE

10 antibodies wherein a murine antibody directed against human IgE (MaE11) was used to provide the CDR regions that were substituted into an IgG1 immunoglobulin framework (rhuMaE25). See also Cacia et al., Biochemistry, 35: 1897-1903 (1996) for studies and further descriptions of E-25.

B. Monoclonal anti-IgE antibody prepared from the culture supernatants of an immortalized human myeloma cell line U266B1 (ATCC TIB 196) using affinity chromatography purification on an isolated anti-IgE antibody (Genentech MAE1). Specifically, five BALB/c female mice, age six weeks, were immunized in their foot pads with 10 μ g of purified IgE in Ribi's adjuvant. Subsequent injections were done in the same manner at one and three weeks after the initial immunizations. Three days after the final injection, the inguinal and popliteal lymph nodes were removed and pooled, and a single cell suspension was made by passing the tissue through steel gauze. The cells were fused at a 4:1 ratio with mouse myeloma P3X63-Ag8.653 (ATCC CRL 1580) in high glucose (DMEM) containing 50% w/v polyethylene glycol 4000. Alternatively, the immunizations were done in a similar manner except that 30 μ g of IgE per injection were used and IgE fragment 315-347 (Kabat) was assayed as a prefusion boost; or injections were given subcutaneously in two doses of 100 μ g and a final booster of 50 μ g, and spleen cells were used for the fusions.

25 The fused cells were then plated at a density of 2x10⁵ per well in 96-well tissue culture plates. After 24 hours HAT selective medium hypoxanthine/aminopterin/thymidine, Sigma, #H0262) was added. Of 1440 wells plated, 365 contained growing cells after HAT selection.

Fifteen days after the fusion, supernatants were tested for the presence of antibodies specific for human IgE using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed as

follows, with all incubations done at room temperature. Test plates (Nunc Immunoplate) were coated for 2 hours with rat anti-mouse IgG (Boehringer Mannheim, #605-500) at 1 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, then blocked with 0.5% bovine serum albumin in phosphate buffered saline (PBS) for 30 minutes, then washed four times with PBS containing 0.05% TWEEN 20™ (PBST). Test supernatants were added and incubated two hours with shaking, then washed four times with PBST. Human IgE (purified from U266 cells as described above) was added at 0.5 µg/ml and incubated for one hour with shaking, then washed four times in PBST. Horseradish-peroxidase-conjugated goat anti-human IgE (Kirkegaard & Perry Labs, #14-10-04, 0.5 mg/ml) was added at a 1:2500 dilution and incubated for one hour, then washed four times with PBST. The plates were developed by adding 100 µl/well of a solution containing 10 mg of o-phenylenediamine dihydrochloride (Sigma, #P8287) and 10 µl of a 30% hydrogen peroxide solution in 25 ml phosphate citrate buffer, pH 5.0, and incubating for 15 minutes. The reaction was stopped by adding 100 µl/well of 2.5 M sulfuric acid. Data were obtained by reading the plates in an automated ELISA plate reader at an absorbance of 490 nm. For one antibody, 365 supernatants were tested and 100 were specific for human IgE. Similar frequencies of IgE specificity were obtained when screening for the other antibodies.

C. Bovine serum Albumin: pI 4.7 and 4.9 (Radola, *Biochim. Biophys. Acta*, **295**: 412-428 (1973))

- Bayer Corp. P/N 81-024-2, "Bovine Albumin, Sulfhydryl Modified" (BSA Mix, blocked)
- ICN Biomedical Inc. P/N 810013, "Albumin Bovine" (BSA Mix, native)
- BSA monomer and dimer prepared in house from Bayer BSA (BSA Monomer and BSA Dimer, respectively)

Chromatography Systems:

Hewlett-Packard 1090™ HPLC

25 Pharmacia™ FPLC

Detection at 215 or 280 nm

Buffers: (see Table I for details)

Purified water

Tris•HCl
 Sodium acetate
 Sodium chloride
 Sodium phosphate
 5 Sodium citrate and citric acid

Sample Preparation:

Samples were diluted with the buffer used for equilibration (indicated in Table I below) to assure pH and conductivity matched starting column conditions. All samples were 0.2- μ m filtered prior to loading.

10 **Chromatography:**

Samples were introduced to the column using either an automatic or manual injector. All runs were performed at room temperature. Fractions were collected manually or with a PHARMACIA FRAC 100TM collector.

15 Chromatographic separation performance was evaluated by comparing elution profiles of BSA stock reagent and purified BSA monomer and dimer; the same was done for IgE and the monoclonal antibodies (MAb). Separation of IgE and MAb from their dimers and multimers was further evaluated by analyzing elution fraction using analytical size-exclusion chromatography. Plots of MAb MW forms vs. Fraction number were created. Recovery of IgE and MAb was determined spectrophotometrically by measuring absorbance at 280nm.

20 **RESULTS:**

The results are summarized in Table I below.

Table I
MONOMER-DIMER/MULTIMER SEPARATIONS

25

RESIN	PROTEIN	PH	EQUILIBRATION BUFFER	ELUTION	COMMENTS
Anion-Exchange					
QSFF TM	MAb	8	Tris-HCl	linear gradient: 0 to 500 mM NaCl	Good separation
				linear gradient: 50 to	Best separation

				200 mM NaCl	
				step gradients to 200, 175, 150, 125 mM NaCl	Separation works
Resource Q™	MAb	8	Tris-HCl		
Resource Q™	U266 IgE	8	Tris-HCl		Removed aggregates and multimers
Resource Q™	BSA Monomer	8	Tris-HCl	linear gradient: 0 to 1M NaCl,	Good separation
	BSA Dimer			linear gradients: 150 to 550, 250 to 550mM NaCl	Excellent separation
	BSA Mix, native				
	BSA Mix, blocked			step gradients: 0.3/0.6, 0.38/0.6, 0.4M/0.6M NaCl	Some separation but fine control required
Resource Q™	BSA Monomer	6	sodium citrate	linear gradient:	Does not bind in citrate pH 6
	BSA Dimer				"
	BSA Mix, blocked				"
Resource Q™	BSA Monomer	6	sodium phosphate	linear gradient: 0 to 0.5M NaCl in 10 CVs	good separations
	BSA Dimer				
	BSA Mix, blocked				
Cation Exchange					
Resource S™	BSA Monomer	6	sodium citrate	linear gradient: 0 to 0.5M NaCl in 10 CVs	does not bind in citrate pH 6
	BSA Dimer				"
	MAb				"
Resource	MAb	6	sodium phosphate	linear gradient: 0 to	equivalent to Q

S TM				0.05M NaCl in 20 CVs	separation
					loaded to 16.5 mg/mL
Resource S TM	BSA Monomer	4.6	NaOAc buffer	linear gradient: 0 to 1M NaCl/40 CVs	proteins somewhat resolved
	BSA Dimer				
	BSA Mix, blocked				
	BSA Monomer	4.3	NaOAc buffer	linear gradient: 0 to 1M NaCl/20 CVs	better resolution than pH 4.6
	BSA Dimer				
	BSA Mix, blocked				
JT Baker CSx TM	BSA Monomer	4.6	NaOAc buffer	linear gradient: 0 to 1M NaCl/12 CVs	proteins somewhat resolved
	BSA Dimer				
JT Baker CSx TM	BSA Monomer	4.3	NaOAc buffer	linear gradient: 0 to 1M NaCl/12 CVs	proteins somewhat resolved
	BSA Dimer				

Separations were evaluated using polymeric polystyrene/divinyl benzene resins (RESOURCE Q and STM), a silica-based resin (JT BAKER CSXTM), and an agarose-based resin (Q-SEPHAROSE FAST FLOWTM; QSFF). While separations were accomplished using any of these resins, separations worked especially well on Q-SEPHAROSE FAST FLOWTM, RESOURCE QTM, and RESOURCE STM. The separation of BSA monomer and dimer from both suppliers looked very similar, suggesting the “Sulphydryl Modified” material from Bayer did not alter the protein such that the species were easier to separate. It can be seen that phosphate buffer at pH 6 worked well, but no protein bound to the cation- or anion-exchange columns when 20 mM citrate buffer at pH 6 was used as equilibration buffer. Citrate buffer would be expected to work for both anion- and cation-exchange at a lower concentration, e.g., about 5 mM.

Recovery of monomeric IgE and MAbs to IgE on anion-exchange resins was typically greater than 90% at greater than 99.5% purity. Figures 1A and 1B show anion-exchange (RESOURCE™ Q) chromatograms in the separation of IgE monomers from dimers and multimers. Figures 2A1 and 2A2 show anion-exchange (RESOURCE™ Q) chromatograms in the separation of anti-IgE MAb monomers from dimers and multimers. Fig. 2B shows an anion-exchange (Q-SEPHAROSE FAST-FLOW™) chromatogram in the separation of anti-IgE MAb monomers from dimers and multimers. SEC (SUPERDEX 200 HR 10/30™) was used as an analytical method to determine the amount of monomer and multimer in samples from the ion-exchange separation, and Figure 2C shows the SEC analysis of fractions from Figure 2B. Separation of BSA monomer from dimer was readily achieved on anion-exchange resins at pH 8 and pH 6. See Figures 3A-C and 4A-C for chromatograms in the separation of BSA monomers from dimers and multimers by anion-exchange (RESOURCE™ Q) at pH 8 (Tris buffer) and at pH 6 (phosphate buffer), respectively.

Recovery and purity of MAb monomer from the cation-exchange resin was comparable to that of the anion-exchange resin. Figures 5A-B show cation-exchange (RESOURCE™ S) chromatograms in the separation of anti-IgE MAb monomers from dimers and multimers at pH 6 (phosphate buffer). Separations of BSA on cation-exchange resins could be performed at pH 4.6 and 4.3, 4.3 being somewhat better. Figures 6A-B show cation-exchange (RESOURCE™ S) chromatograms in the separation of BSA monomers from dimers and multimers at pH 4.3 (acetate buffer).

In summary, mixtures of polypeptide mers were subjected to cation- or anion-exchange chromatography using a variety of resins and under a variety of pH and elution salt conditions, and successful separation was achieved. Based on results from four proteins with basic and acidic isoelectric points (two IgG₁ MAbs, IgE and serum albumin), the method demonstrates general applicability to separation of polypeptide monomers from their dimers and multimers.

WHAT IS CLAIMED IS:

1. A method is disclosed for separating a polypeptide monomer from a mixture comprising dimers or multimers or both, wherein the method comprises applying the mixture to a cation-exchange or anion-exchange chromatography resin in a buffer, wherein if the resin is cation-exchange, the pH of the buffer is about 4-7, and wherein if the resin is anion-exchange, the pH of the buffer is about 6-9, and eluting the mixture at a gradient of about 0-1 M of an elution salt, wherein the monomer is separated from the dimers or multimers or both present in the mixture.
2. The method of claim 1 wherein the polypeptide is an antibody or a serum albumin.
3. The method of claim 2 wherein the antibody is anti-IgE, anti-IgG, anti-Her-2, anti-CD11a, anti-CD18, anti-CD20, or anti-VEGF.
4. The method of claim 2 wherein the serum albumin is bovine serum albumin.
5. The method of claim 1 wherein the ion-exchange resin is a cation-exchange resin.
6. The method of claim 1 wherein the ion-exchange resin is an anion-exchange resin.
7. The method of claim 1 wherein the gradient is linear.
8. The method of claim 1 wherein the gradient is stepwise.
9. The method of claim 1 wherein the elution salt is a sodium salt.
10. The method of claim 9 wherein the elution salt is sodium chloride.
11. The method of claim 1 wherein the gradient is from 0 to 500 mM elution salt.

12. The method of claim 1 wherein the gradient is from 50 to 200 mM elution salt.
13. The method of claim 1 wherein the gradient is from 0 to 50 mM elution salt.

SEPARATION OF POLYPEPTIDE MONOMERS

Abstract of the Disclosure

A method is disclosed for separating a polypeptide monomer from a mixture comprising dimers and/or multimers. The method comprises applying the mixture to either a cation-exchange chromatography resin or an anion-exchange chromatography resin and eluting the mixture at a gradient of about 0-1 M of an elution salt, wherein the monomer is separated from the dimers and/or multimers present in the mixture.

Figure 1

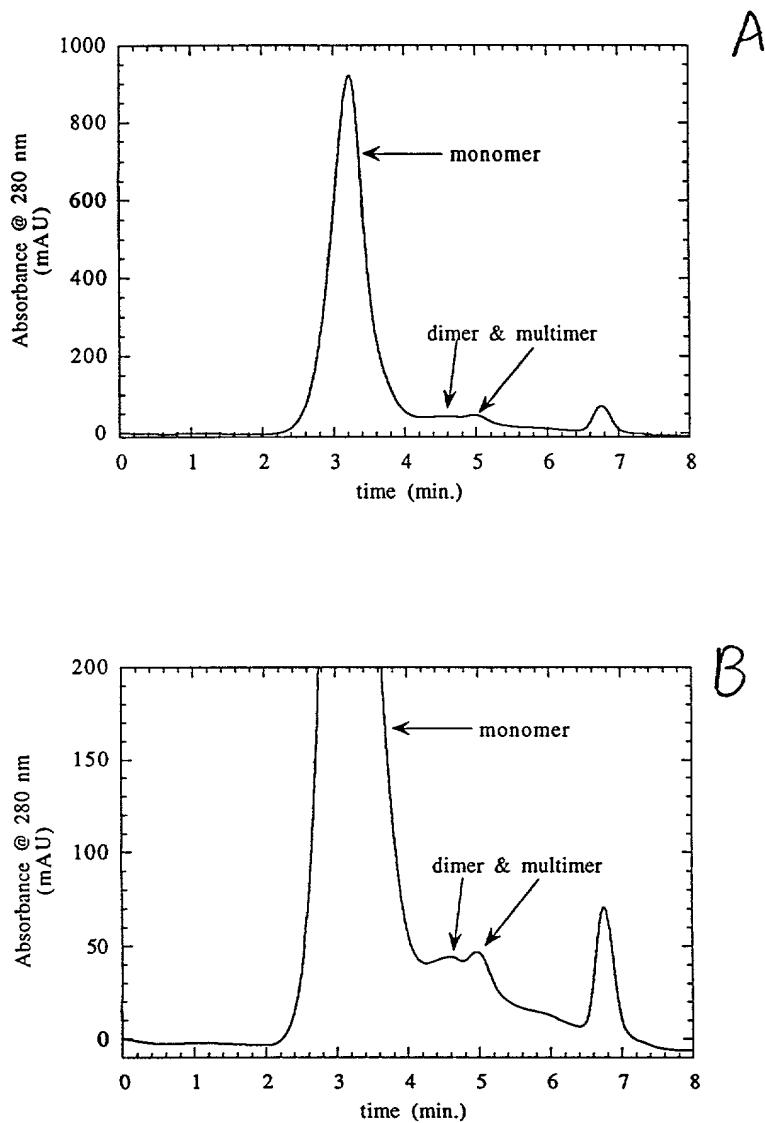


Figure 2

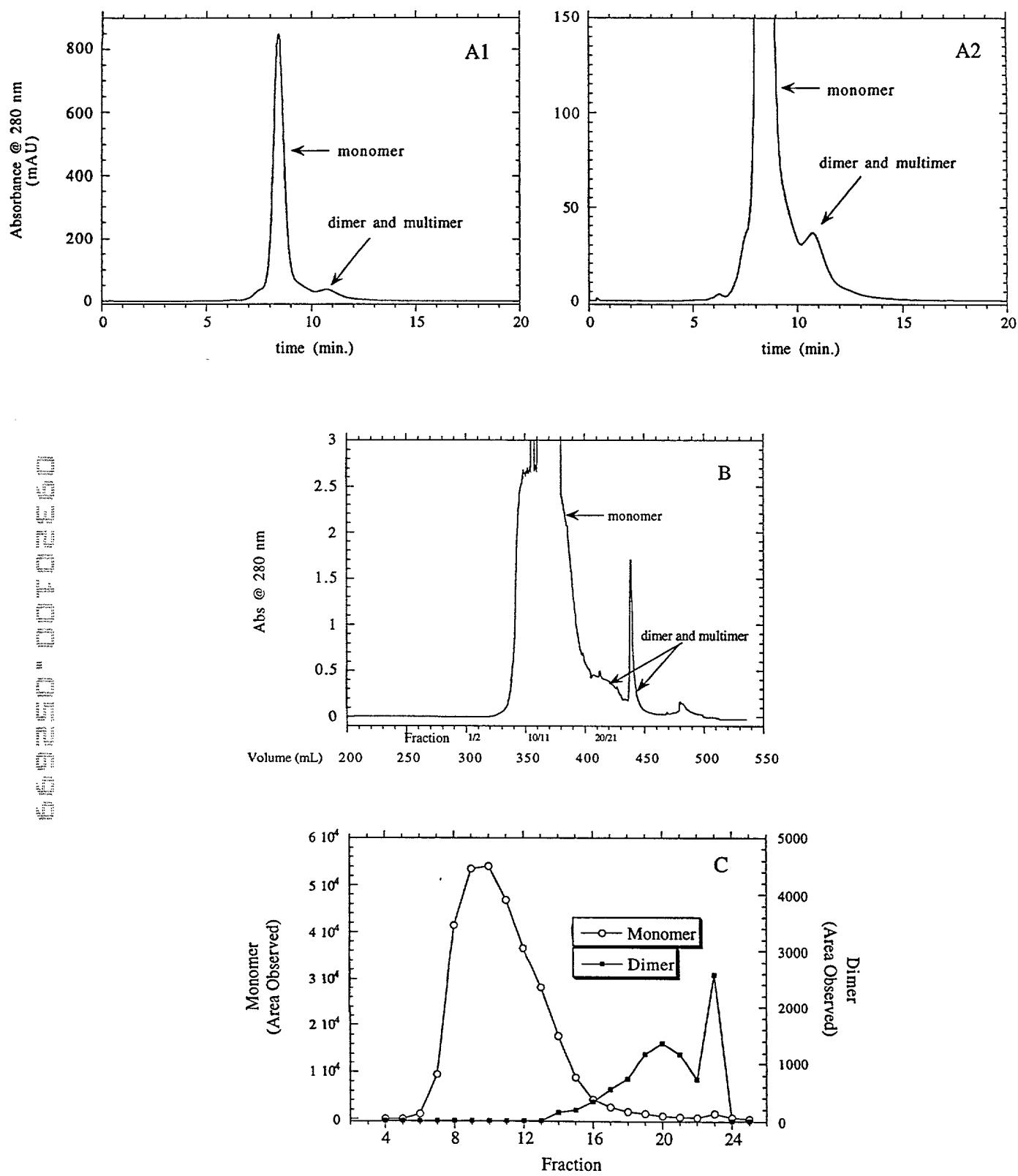


Figure 3

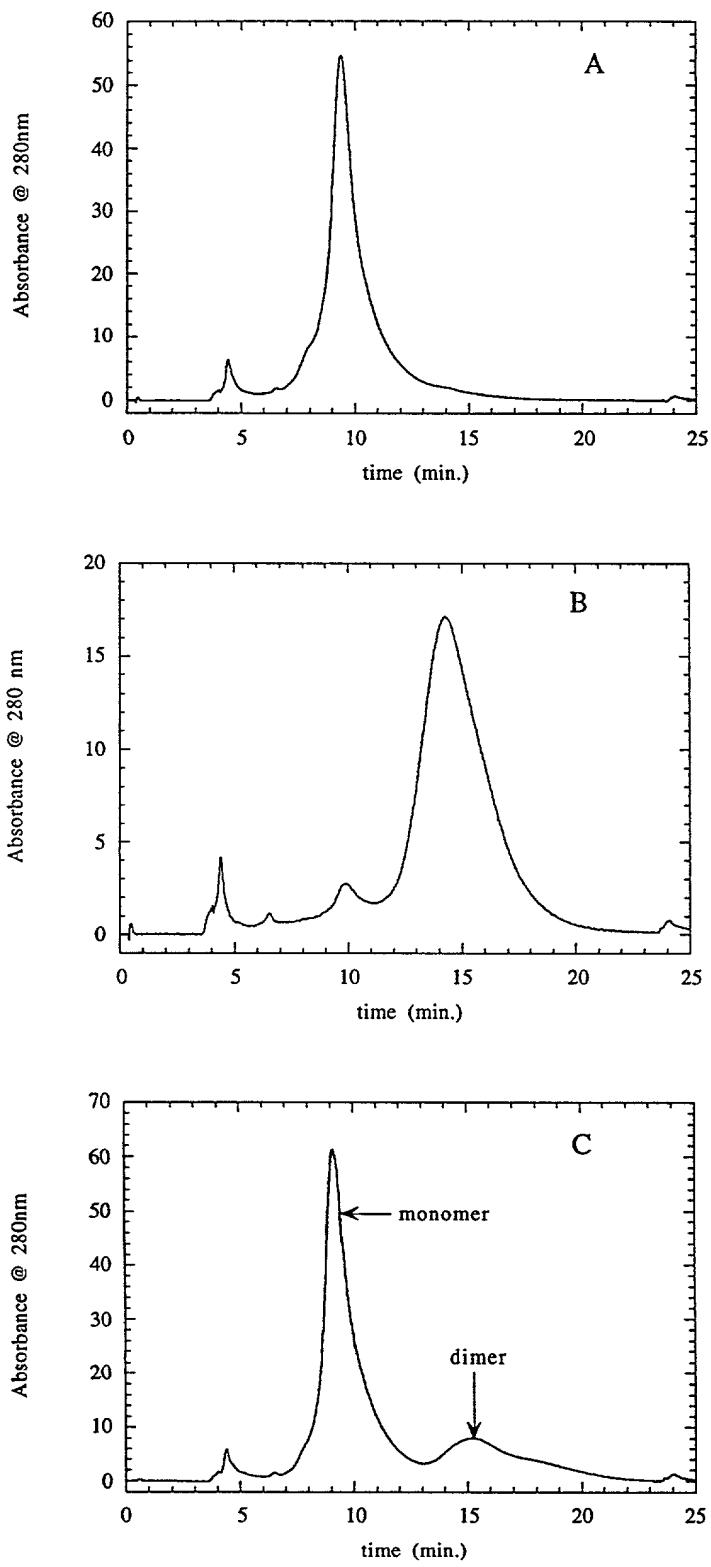


Figure 4

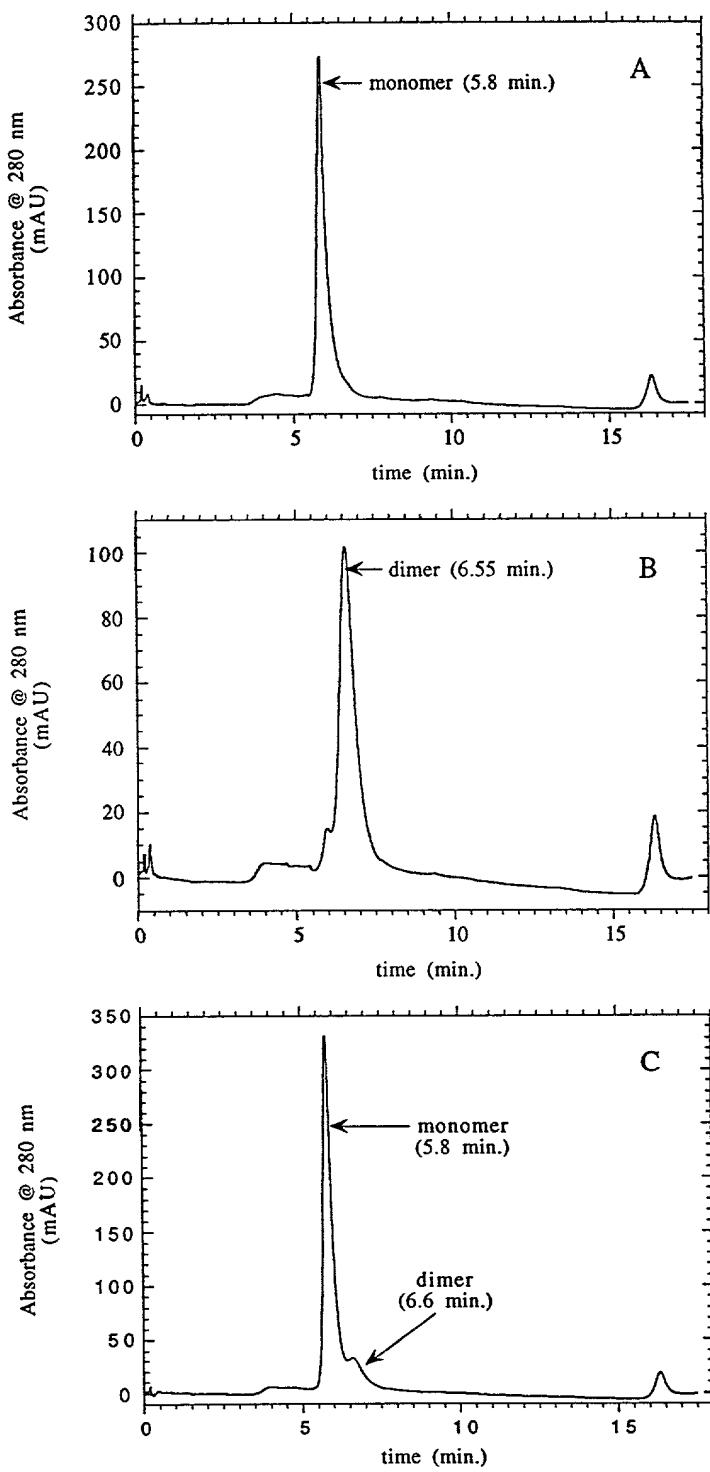


Figure 5

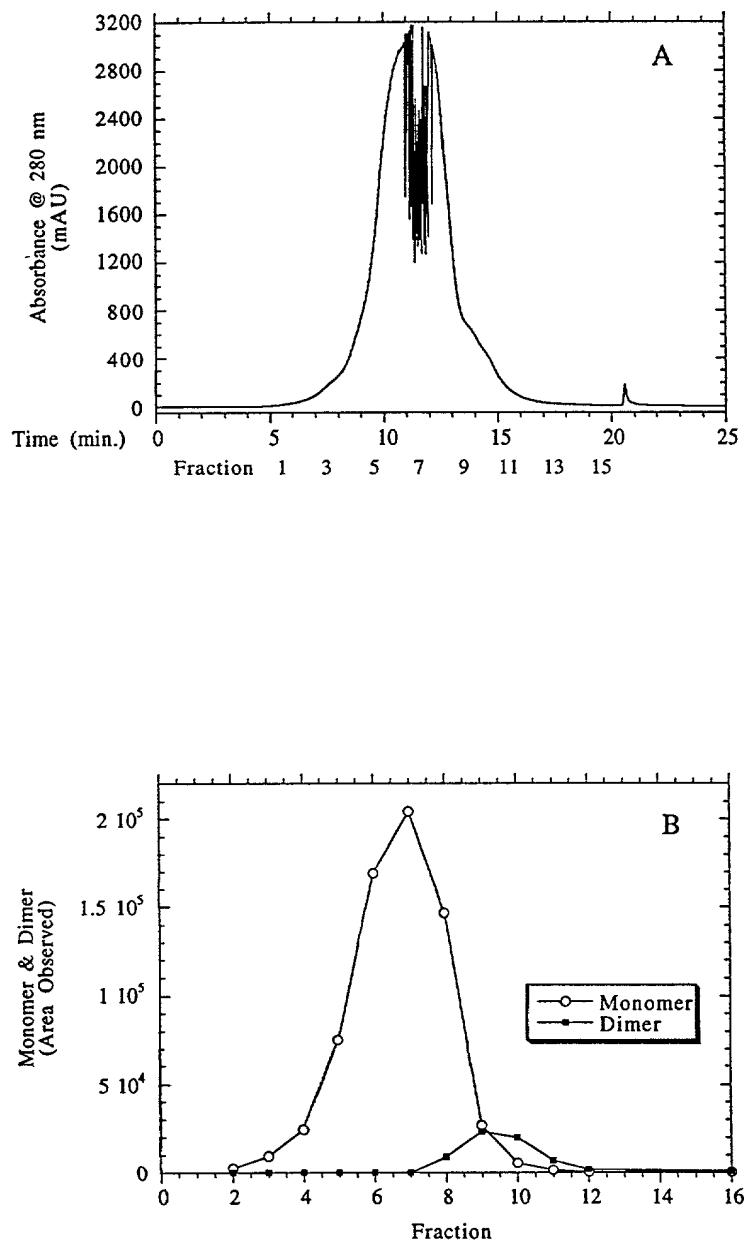
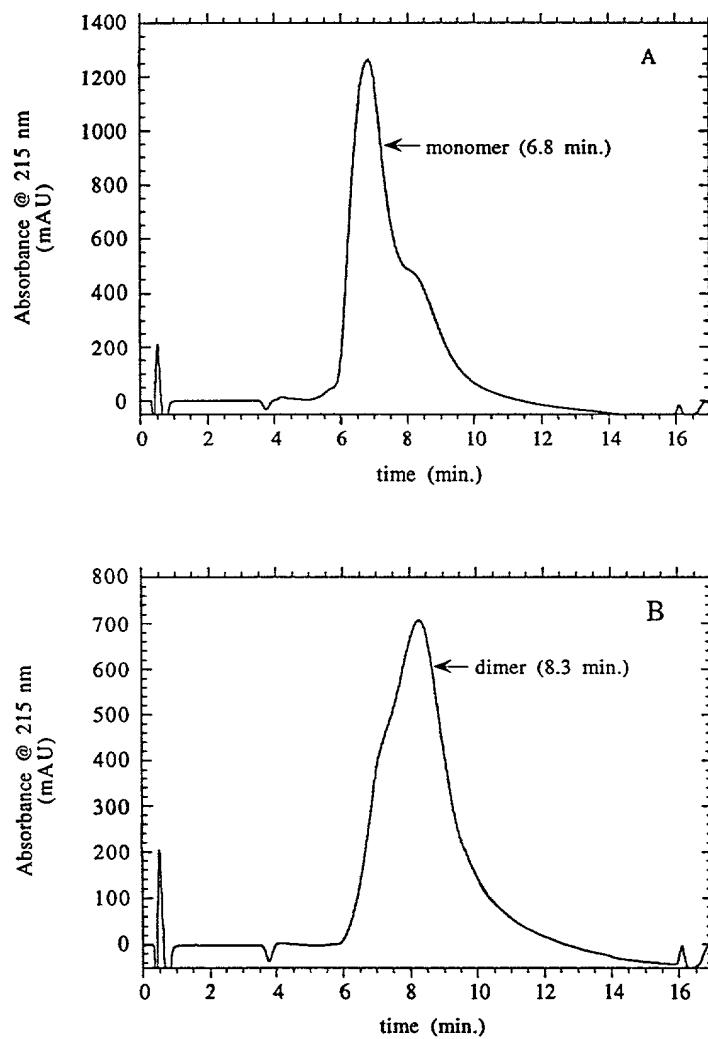


Figure 6



EXPRESS MAIL NO: EM 168 883 029 US
DATED: 26 May 1999

Docket No. P1363R1

**COMBINED DECLARATION FOR PATENT APPLICATION
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SEPARATION OF POLYPEPTIDE MONOMERS

the specification of which (check one) X is attached hereto or _ was filed on _ as Application Serial No. _ and was amended on _ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate have a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s):	Priority Claimed
	<u>Yes</u> <u>No</u>

Number	Country	Day/Month/Year Filed
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I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional applications(s) listed below:

60/087,602	June 1, 1998
Application Ser. No.	Filing Date

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
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Application Ser. No.	Filing Date	Status: Patented, Pending, Abandoned
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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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